## REMARKS

Favorable reconsideration of this application in light of the preceding amendments and the following remarks is respectfully requested.

Claims 1, 3 and 7 are now pending in the application. Claims 2, 4, 5, 8, 10-12 having been cancelled herein, no claims having been added. Minor amendments have been made to the specification and claims to simply overcome the objections to the specification and rejections of the claims under 35 U.S.C. § 112. The Examiner is respectfully requested to reconsider and withdraw the rejection(s) in view of the amendments and remarks contained herein.

**Specification**

The Specification stands objected to for the introduction of new matter as alleged by having deleted the phrase "or green fluorescent protein from pEDFP-1" at paragraph [0020] and the phrase "or green" at paragraph [0021] of the specification." (Action at 2). The Applicant has cancelled the new matter by amending the specification as recommended by the Examiner. The Applicant requests, therefore, that these objections be reconsidered and withdrawn accordingly.

**Claim Objections**

Claims 1, 2, 3, 10 and 12 stand objected to for various informalities as detailed by the Examiner, Action at 3 & 4. The Applicant submits that the amendments to and/or cancellation of the claims reflected above are sufficient to overcome and/or render moot these objections. The Applicant requests, therefore, that these objections be reconsidered and withdrawn accordingly.

**Double patenting**

Examiner submits that if Claim 10 were found allowable, claim 12 would be objected to under 37 CFR 1.75, Action at 4. The Applicant submits that the cancellation of Claims 10 and 12 are sufficient to overcome and/or render this Double Patenting objection moot.

**Rejection Under 35 U.S.C. § 112, second paragraph**

Claims 1, 2, 4, and 7-8 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to point out and distinctly claim the subject matter which Applicant regards as the invention. ( Action at 5). This rejection is respectfully traversed.

Applicant respectfully submits that in this instance the α-actin promoter as recited in Claim 1 i.e. "α-actin gene promoter of golden zebrafish" is sufficiently clear and definite to one of ordinary skill in the art. To expedite prosecution and reduce the number of issues for disposition, the Applicant has amended Claim 1 to recite "a golden zebrafish α-actin promoter" to particularly point out and distinctly claim a promoter associated with α-actin of golden zebrafish.

Claim 7 stands rejected as allegedly being unclear for failing to define the metes and bounds of the term "systemic". The Applicant has amended Claim 7 herein, and respectfully submits that the amendment to Claim 7 makes clear that the red fluorescence exhibited by an adult golden zebrafish is that fluorescence expressed in the skeletal muscle homogenously. (Sub. Spec. at paragraphs [0005] & [0016]).

The Applicant submits, therefore, that the amendments to the claims reflected herein and the cancellation of Claims 2, 4, 8 and 10 above are sufficient to overcome these rejections and request that they be reconsidered and withdrawn accordingly.

**Rejection Under 35 U.S.C. § 103**

Claims 5, 8 and 12 remain rejected and Claims 3-4, 6, 7 and 9-11 are presently rejected under 35 U.S.C. 103(a) as being unpatentable over Hsiao et al. [2001] herein "Hsiao", in view of Carvan et al. [2000] herein "Carvan" and further in view of Finley et al. [Biotechniques, 31:66-72, July 2001] herein "Finley". This rejection is respectfully traversed.

Applicant notes that Claims 3 and 7 remain pending, having cancelled Claims 2, 4, 8, and 10-12 herein. Claim 3 is drawn to a method of producing an adult golden zebrafish with systemic red fluorescence comprising: (a) constructing a plasmid including a first ITR, a CMV promoter, a gene encoding DsRed, S40 poly A and a second ITR; (b) replacing the CMV promoter with an $\alpha$-actin gene promoter of golden zebrafish to produce a new plasmid construct in which the $\alpha$-actin gene promoter is operably linked to the gene encoding DsRed; (c) linearizing the new plasmid construct; (d) microinjecting the linearized new plasmid construct into fertilized golden zebrafish eggs to obtain microinjected eggs; (e) incubating the microinjected eggs for at least 24 hours to form embryos; (f) selecting an embryo exhibiting red fluorescence; and (g) cultivating the selected embryo to maturity to produce an adult golden zebrafish having skeletal muscle that exhibits red fluorescence.

Although Hsiao teaches the use of an EGFP fluorescent protein gene in a linearized plasmid having flanking ITRs, an $\alpha$-actin promoter, and SV40 poly A, Hsiao does not teach or suggest the use of a red fluorescent protein gene construct or any other fluorescent protein

construct that may impart new and novel fluorescence features to ornamental fish, including the enhanced red fluorescence that can be viewed unaided. Moreover, Hsiao fails to disclose or suggest red fluorescence that is systemic and homogenously distributed throughout the zebrafish musculature.

The deficiencies of Hsiao are not cured by Carvan. Carvan's use of a fluorescent gene construct for transient reporter function would not be sufficient for one of ordinary skill in the art to expect that the same construct could be used to produce transgenic zebrafish. The fluorescence expression in Carvan is unrelated to stable fluorescence, but is directed to monitoring pollution induced expression of fluorescence which is completely different. The presently claimed gene fragment lacks any of the response enhancers found in Carvan. As Carvan specifically mentions: "Other laboratories have had the same difficulties in sustaining transgene expression beyond the F2 generation in zebrafish for reasons not known, but possibly due to an efficient genome surveillance system in this species." Carvan at page 143, par. 3. Clearly, Carvan is noting and concurring with others in the field with the general idea that it is difficult to make transgenic fluorescent zebrafish. This reference lends credence to the Applicant's assertion that the presently claimed gene fragment and method of making adult golden zebrafish with systemic red fluorescence is not rendered obvious by the references cited in the Action herein.

The Examiner contends that the Applicant's rebuttal to the Examiners assertion that "visible systemic fluorescence in adult fish is an inherent feature of transgenic fish that incorporate a gene encoding a fluorescent gene product." (Action at 11) by citing Opsahl et al. is unpersuasive. In support of the contention, the Examiner contends that "Opsahl et al. is relevant to mouse species and not to fish strains." Furthermore, the Examiner alleges that "[T]he effects

on promoter activity would not be commonplace and predicted to occur with most promoters and position effect variegation is easily overcome by generating additional lines of transgenic fish."

Applicant respectfully submits that the Opsahl et al. reference was asserted to provide evidence that suggests that even in related species and strains, subtle genetic differences can lead to variations in transgene insertion, maintenance, and expression. Although, evidence was provided for differences occurring between mouse strains as disclosed by Opsahl et al., the Examiner has provided no evidence why the teachings of Opsahl et al. could not be directly applied to different strains of zebrafish. In addition, the Applicant submits herein evidence that the field of transgenics and transgene expression has in the past and continues, at least to the date of filing the present application, is unpredictable with respect to successful, stably integrated transgenes. The mere substitution of one promoter, for example Xenopus EF1α promoter for an α-actin promoter would not guarantee success in creating a stably integrated fluorescent protein gene as exemplified in the present application.

Transgene position effects are problematic in developing stably integrated transgenes. As recited in Feng et al., Mol. & Cell Biol. 21:298-309 (2001) (a courtesy copy is provided herewith), "Stably integrated transgenes are often poorly expressed because of position effects that are caused by the influence of the site of chromosomal integration." (Feng, page 298). Furthermore, integration sites can be random and bear no relationship to the sequence injected. Often multiple copies of the injected sequence insert together at the same time and rearrangement or deletion of the sequence can occur. Moreover, transgenes can become rearranged, or inserted at locations where they are inactivated (e.g. heterochromatin) or come under the control of endogenous regulatory elements, (e.g. cytosine DNA methylation and

chromatin structure modification), thus making transgene expression difficult, unpredictable and non-obvious over the cited references.

The Applicant submits that Finley does not cure the deficiencies of Hsiao and Carvan. Finley teaches the use of red fluorescent reporter constructs that can be injected into zebrafish embryos. The reporter constructs in Finley using DsRed gene fragments were injected into embryos for transient expression. Finley states "We anticipate the use of multiple fluorescent proteins to facilitate insertional mutagenesis, enhancer and gene-trapping techniques, and protein-protein interaction investigations using fluorescently labeled protein constructs in single cells." (emphasis added) (Finley page 66). Finley does not teach, disclose or suggest the use of DsRed for stable integration into zebrafish systemic skeletal muscle. Finley teaches a very different mechanism of utilizing the gene fragment, or method of operation using the DsRed fluorescent sequence as compared to the exemplified embodiments. The Applicant's method of producing stable red fluorescence transgenic zebrafish via integration of the gene fragment recited in Claim 3 into the genome of the zebrafish results in stable transgenic zebrafish whose fluorescence can be seen even without the aid of a confocal microscope. The method of producing Applicant's adult golden zebrafish with systemic red fluorescence is not rendered obvious by Finley, because Finley only teaches the use of fluorescent protein expression in zebrafish for insertional mutagenesis, enhancer and gene trapping techniques and protein-protein interactions. (Finley, page 66, Col. 2). Furthermore, the red fluorescent protein gene was under the control of Xenopus EF1α promoter, a promoter that is operationally different to the actin promoters used in the present application and may not function as disclosed in the exemplified embodiments, given the unpredictability or promoter activity in a different strain of zebrafish.

Finley fails to teach or suggest a method for producing an adult golden zebrafish with systemic red fluorescence of the musculature as disclosed in the exemplified embodiments, nor does Finley teach or suggest how a transgenic zebrafish could be constructed to yield the same systemic muscle red fluorescence as exemplified in the present application. One of ordinary skill in the art would not have a reasonable expectation of success in combining the teachings of Carvan and Finley with those of Hsiao, since no reference either alone or in combination teach how to produce an adult golden zebrafish with systemic red fluorescence transgenically.

The Applicant submits that the present claims are directed to the successful generation of viable red fluorescent adult zebrafish that were not taught or suggested in the prior art. The Applicant maintains, therefore, that achieving expression of a red fluorescent gene product in adult zebrafish is both novel and not obvious.

Claim 1 stands rejected under 35 U.S.C. 103(a) as being unpatentable over Hsiao et al. [2001], in view of Finley et al. [Biotechniques, 31:66-72, July 2001].

Claim 1 as amended recites "a gene fragment comprising, in an upstream to downstream order the operatively linked regions (1) a first inverted terminal repeat (ITR) of adeno-associated virus; (2) a golden zebrafish α-actin promoter; (3) a gene encoding DsRed; (4) SV40 poly A and (5) a second inverted terminal repeat (ITR) of adeno-associated virus."

The Examiner alleges "that it would have been obvious to one of ordinary skill in the art at the time of filing to combine the technology taught by Hsiao of using ITR elements to enhance fluorescent reporter gene expression in transgenic fish with the teachings of Finley regarding the use of DsRed as a fluorescent reporter." (Action at 15).

The Applicant respectfully disagrees with the Examiner's conclusion that "one of ordinary skill in the art would have been motivated to combine these teachings..."

The Applicant also contends that while Finley did teach the use of DsRed in wild-type zebrafish, the Applicant notes that red fluorescence expression in zebrafish disclosed by Finley is driven by Xenopus EF1α promoter and that Finley only recorded fluorescent phenotype at early developmental stage up to 24-dpf embryo and did not provide any data with respect to adult fish. In addition, the Applicant contends that red fluorescence expression of DsRed driven by Xenopus EF1α promoter is more in discrete or patchy groups in embryo (as detailed in Finley et al., Biotechniques 31:66-72) rather than in a systemic expression pattern as in present invention. Finley teaches the use of DsRed in a construct having a different promoter that may not appreciably function in the same manner, or to the extent as the promoter used in the present application. Such a deficiency would defeat any reasonable expectation of success for combining the linearized plasmid of Hsiao with the construct of Finley to stably transform zebrafish with systemic red fluorescence as disclosed in the present application.

The Applicant has prepared the following TABLE to illustrate the differences between the pending claims and the teachings of the applied references.

TABLE

| Promoter | The present application | Hsiao | Carven | Finley |
|---|---|---|---|---|
| Promoter | α-actin promoter | α and β-actin promoter | CMV promoter EF1α promoter | Xenopus EF1α promoter |
| Strain of Zebrafish | golden | leopard | long-fin and golden double mutant | wild-type |
| Stage of zebrafish with fluorescence Type of fluorescence | larva and adult DsRed from Coral | Larval and adult fish EGFP **green** fluorescence | Larval and adult fish GFP **green** fluorescence | Larval fish With **red** fluorescence |
| Purpose | Ornamental use | Academic use | Aquatic pollution detection | Academic use |

Applicant also respectfully submits that in the determination of non-obviousness, the Examiner must also take into consideration secondary factors indicative of non-obviousness such as commercial success and failure of others to produce the claimed transgenic fish or methods of making such ornamental fish. The claimed gene fragment when incorporated into the genome of the golden zebrafish results in unexpectedly stable and uniform red fluorescence of skeletal muscle, previously unreported. It is the Applicant that has successfully developed a gene fragment comprising DsRed and a method capable of stable integration into zebrafish providing systemic skeletal red fluorescence expression.

The Applicant respectfully requests, therefore, that the present rejection under 35 U.S.C. 103(a) of Claims 1, 3 and 7 be reconsidered and withdrawn. Applicant notes that rejection of Claims 2, 4-6, 8-11 under 35 U.S.C. 103(a) is deemed moot in view of the cancelled Claims above.

**Conclusion**

In view of the above remarks and amendments, the Applicant respectfully submits that each of the pending objections and rejections have been addressed and overcome, leaving the present application in condition for allowance. A notice to that effect is respectfully requested.

If the Examiner believes that personal communication will expedite prosecution of this application, the Examiner is invited to contact the undersigned.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies to charge any underpayment or non-payment of any fees required under 37 C.F.R. §§ 1.16 or 1.17, or credit any overpayment of such fees, to Deposit Account No. 08-0750, including, in particular, extension of time fees.

Respectfully submitted,

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By: _____

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